Improving and accelerating the differentiation and functional maturation of human stem cell-derived neurons: role of extracellular calcium and GABA.

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Running title: Enhancing functional neuronal differentiation of stem cells

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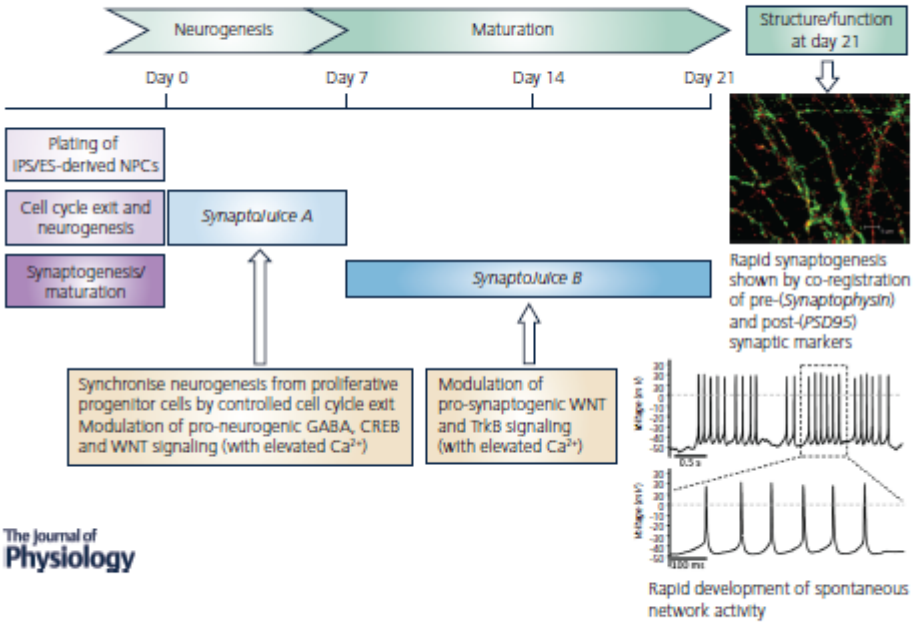
Abstract

Neurons differentiated from pluripotent stem cells using established neural culture conditions often exhibit functional deficits. Recently, we have developed enhanced media which both synchronise the neurogenesis of pluripotent stem cell-derived neural progenitors and accelerate their functional maturation; together these media are termed *SynaptoJuice*. This pair of media are pro-synaptogenic and generate authentic, mature synaptic networks of connected forebrain neurons from a variety of induced pluripotent and embryonic stem cell lines. Such enhanced rate and extent of synchronised maturation of pluripotent stem cell-derived neural progenitor cells generates neurons which are characterised by a relatively hyperpolarized resting membrane potential, higher spontaneous and induced action potential activity, enhanced synaptic activity, more complete development of a mature inhibitory $GABA_A$ receptor phenotype and faster production of electrical network activity when compared to standard differentiation media. This entire process - from pre-patterned neural progenitor to active neuron - takes 3 weeks or less, making it an ideal platform for drug discovery and disease modeling in the fields of human neurodegenerative and neuropsychiatric disorders, such as Huntington's disease, Parkinson's disease, Alzheimer's disease or Schizophrenia.

Illustrated abstract. *Rationale and basic protocol for accelerating neuronal maturation using SynaptoJuice.* Neural precursor cells (NPCs) that have been generated from either human embryonic (ES) stem cells or human induced pluripotent stem (iPS) cells are plated at day 0 in SynaptoJuice A. This first medium has been designed to synchronise neurogenesis, by foricng NPCs to exit the cell cycle, whilst increasing GABA-induced Ca^{2+} influx. At day 7, and thereafter, *SynaptoJuice A* is replaced by *SynaptoJuice B* which is designed to promote synaptogenesis via WNT and TrkB signaling. At Day 21, the resulting neurons demonstrate punctate co-registration of synaptic markers (shown here are synaptophysin and PSD95) and synchronised electrical activity (shown here is an exemplar spontaneous train of action potentials).



Key words: Stem cell; Calcium channel; Astrocyte; GABA receptor; Patch-clamp; Neural circuit

Author profiles

Paul J. Kemp completed his DPhil in Physiology in the Department of Human Anatomy at the University of Oxford in 1990. Since then he has held academic positions in the University of Dundee, the University of Southern California and the University of Leeds. Since 2004, he has been Professor of Physiology at Cardiff University School of Biosciences where his group works on gas sensing by ion channels, novel asthma therapeutics (with Daniela Riccardi) and fate determination of human pluripotent stem cells (with Nicholas D Allen).

Vsevolod Telezhkin was trained in the Bogomoletz Institute in Kiev where he obtained his PhD in 2003. Following several successful post-doctoral positions at the Universities of Vermont, Bath and Cardiff he worked at University College London with Professor David Brown, FRS, with whom he published a series of important papers on regulation of M-channels by lipids. Currently, he is an associate at Cardiff University where his main interest is in the role of M-channels in pluripotent stem cell differentiation and neuronal maturation.

Introduction

Since the original isolation and characterization in 1998 (Thomson *et al.*, 1998) of human embryonic stem cells (ESCs), followed by the discovery some ten years later of human somatic cell reprogramming into induced pluripotent stem cells (iPSCs) (Takahashi *et al.*, 2007), pluripotent stem cells have been regularly hailed as a panacea for understanding and, potentially, treating an ever increasing list of human disorders, providing the raw material for cell replacement therapies, disease modeling and drug screening. Review of clinical trials shows that pluripotent stem cells have been employed with variable success as cell replacements in a variety of disorders, either directly or via population of artificial support matrices, including tracheal replacement (Jungebluth *et al.*, 2011) retinal dysfunction (Zarbin, 2016), spinal cord injury (Lukovic *et al.*, 2014) and several neurodegenerative diseases (Levy *et al.*, 2016). Unlike replacement therapies, which require the use of either the pluripotent stem cells themselves or pre-patterned/pre-specified cell lineage-specific precursors, disease modeling and drug screening frequently have the explicit requirement for a population of terminally differentiated cells of the type affected in the disease in question. This requirement has led to the rapid and spirited development of protocols aimed at directing differentiation of pluripotent stem cells to correctly specified cells. Such developments have been particularly competitive and often controversial in the field of neuroscience where the scope for mixed populations of neurons is high but often undesirable when the exact cellular composition *in vitro* may not yet be fully defined.

Independently of specification of a particular neuronal cell type, it is absolutely imperative that differentiated neurons demonstrate appropriate cell biological characteristics and functional activities. In the field of neuronal differentiation, the efficiency and efficacy of the majority of protocols is assessed using an array of established cell-specific markers employing a combination of immunocytochemistry (ICC), reverse transcription polymerase chain reaction (RT-PCR) and, more recently, RNA sequencing (RNAseq) and proteomic analyses. However, except in a handful of exceptional studies (Johnson *et al.*, 2007; Song *et al.*, 2013; Livesey *et al.*, 2014; Telias *et al.*, 2014; Bardy *et al.*, 2015), integration of functional assays of neuronal function is rare and, where reported, is normally restricted to single examples of induced action potentials and/or miniature synaptic potentials, with no further analyses. This relative dearth of functional information regarding appropriate neuronal activity is troublesome when such protocols are used to develop meaningful disease models and high-throughput drug screens. In other words, it is unclear whether particular protocols consistently generate functionally active neurons whose activity has not been robustly characterised or, more worryingly, that the protocols do not produce neurons with robust

electrophysiological activity characteristic of the neurons being studied. Indeed, we and others have found that published neuronal differentiation protocols generate neurons which express the appropriate marker molecules but which often fail to generate authentic neuronal activity with any consistency across the population. With this in mind, recent work in several laboratories, including our own, has focused on improving basic neuronal function during the *in vitro* differentiation process using small molecule regulators of specific cell biological cascades and manoeuvres aimed at mimicking physiological and environmental cues which are known to be important during neuronal differentiation *in vivo* in order to improve function and to stimulate synaptogenesis and consequent neuronal network activity *in vitro*.

Regulation of physiological pO_2 and of extracellular free ionised calcium and their potential roles in neuronal differentiation.

Neuronal specification and differentiation *in vivo* occurs prenatally in an environment which is markedly different from that postnatally (Mohyeldin *et al.*, 2010). Of particular significance is the partial pressure of oxygen (pO_2) which rapidly changes from around 20-30 mmHg to 150-160 mmHg following the first breath of the emerging neonate (Ward, 2008). Such low pO_2, or relative hypoxia, has been successfully employed to improve the efficiency of the generation of iPSCs (Bilican *et al.*, 2012; Hawkins *et al.*, 2013) and a handful of studies have investigated the effect of such relative hypoxia on subsequent neutralisation, specification, and differentiation of neurons (*e.g.*(Studer *et al.*, 2000; Bilican *et al.*, 2014))

Less well appreciated is the parallel switch in extracellular free ionised Ca^{2+} concentration ($[Ca^{2+}]_o$) which occurs in the perinatal period. Thus, human fetal plasma $[Ca^{2+}]_o$ is ~1.6-1.7 mM (Kovacs & Kronenberg, 1997) and this is rapidly reduced within 24 hours postnatally to the mean adult level of 1.1-1.3 mM (Brown, 1991); the relative hypercalcaemia of the developing fetus compared to maternal levels is retained independently of any changes in the maternal $[Ca^{2+}]_o$ (Kovacs *et al.*, 1998). In health, the relatively low postnatal $[Ca^{2+}]_o$ persists and is strictly regulated within a very narrow range by the action of parathyroid hormone on the kidney, intestine and bone; a homeostatic process which is critically dependent upon the activation by $Ca^{2+}{}_o$ of the extracellular Ca^{2+}-sensing receptor (CaSR) expressed in the parathyroid gland (Brown *et al.*, 1993; Riccardi & Kemp, 2012). The striking transition from high prenatal to low postnatal $[Ca^{2+}]_o$ is mediated by the CaSR (Kovacs *et al.*, 1998).

Many commercially available tissue culture media contain $[Ca^{2+}]_o$ broadly embracing the mean value of postnatal $[Ca^{2+}]_o$ in humans of 1.1-1.3 mM (Brown, 1991). With this knowledge in mind, it is

puzzling that the majority of neuronal specification and differentiation protocols employ these standard culture media which contain relatively low $[Ca^{2+}]_o$, or hypocalcaemic, conditions for the developing fetus. Indeed, where studied, the effect of increasing $[Ca^{2+}]_o$ from adult to fetal values is permissive for neurite outgrowth of prenatal primary neurons isolated from the sympathetic cervical ganglion *in vitro* (Vizard *et al.*, 2008).

Role of astrocytes, and astrocyte-conditioned medium, in neuronal differentiation.

It has long been recognised that co-culture of primary neurons with astrocytes promotes neurogenesis and enhances synaptogenesis both directly (Pfrieger & Barres, 1997; Ullian *et al.*, 2001; Hama *et al.*, 2004; Ullian *et al.*, 2004) and indirectly via secreted factors (Chang *et al.*, 2003; Ullian *et al.*, 2004; Chou *et al.*, 2008). Furthermore, maturation of human stem cell-derived neurons can be enhanced by co-culture with astrocytes (Johnson *et al.*, 2007) or astrocyte conditioned medium (ACM) (Rushton *et al.*, 2013; Tang *et al.*, 2013). Indeed, we have shown that ACM has the effect of accelerating the functional maturation rate of iPSC-derived neurons during differentiation by hyperpolarizing the resting membrane potential, thereby increasing the spontaneous activity of the developing neurons, often generating neurons with complex, biphasic patterns of excitability (Rushton *et al.*, 2013). Such relative hyperpolarization, which is seen during differentiation of human iPSCs to neurons *in vitro*, appears to be physiologically relevant as evidenced by the fact that it can also be observed *in vivo*, where we have preliminary evidence to show that developmentally regulated expression of Kv7.2/7.3 (M-type) currents appears to underlie a gradual hyperpolarization of striatal neurons during the 2^{nd} and 3^{rd} trimesters of mouse gestation (Telezhkin *et al.*, 2014).

Previous observations have reported that there are increases in Ca^{2+} channel expression and/or function early in stem cell-derived and native NPC differentiation (Arnhold *et al.*, 2000; Mazzanti & Haydon, 2003; D'Ascenzo *et al.*, 2006). This led us to investigate voltage-gated Ca^{2+} entry, and the potential role of Ca^{2+} channels, in the ACM-evoked enhancement of human iPSC neuronal differentiation (Rushton *et al.*, 2013). Differentiation in ACM evoked a robust enhancement of depolarization-evoked Ca^{2+} entry, principally via L-type, N-type and R-type Ca^{2+} channels; with little contribution of P/Q-type channels (Aga toxin-sensitive) (Figure 1A). Consistent with the selective upregulation of Ca^{2+} influx through voltage-gated Ca^{2+} channels, chronic blockade of either L-type (nifedipine), N-type (conotoxin), or R-type (SNX-482) Ca^{2+} channels during the differentiation process resulted in complete abolishment of the ACM-evoked enhancements of spontaneous activity and significantly depolarized resting membrane potential (Figure 1B). Furthermore, similar

diminutions were seen when ionotropic $GABA_A$ receptors were inhibited using bicuculline, the first suggestion that a major contributor to Ca^{2+} influx during differentiation might be provided by excitatory GABAergic synaptic input in these developing neuronal networks (Figure 1B). However, it should be noted that no direct comparison of $[Ca^{2+}]_i$ in the two conditions was attempted in these studies.

Roles for $GABA_A$ receptors and extracellular Ca^{2+} in neuronal differentiation.

The fact that the robust enhancement of function supported by ACM was characterised by increased Ca^{2+} influx through specific Ca^{2+} channels, and that it could be attenuated by their inhibition, suggested that the mechanism of the ACM effect might involve chronic augmentation of intracellular free ionised calcium concentration ($[Ca^{2+}]_i$) via $GABA_A$-dependent excitation. In order to test this possibility, we attempted to mimic, at least early in the differentiation process, the permissive effects of ACM by chronically raising the extracellular GABA concentration, and the $[Ca^{2+}]_o$ to a level similar to that measured in fetal plasma, namely 1.8 mM (Kovacs & Kronenberg, 1997). These manoeuvres allowed the study of the efficiency and efficacy of differentiation of iPSC-derived neural progenitor cells (NPCs) to neurons under conditions where the $[Ca^{2+}]_i$ was predicted to have been increased tonically (Rushton *et al.*, 2013), although it must be noted that no direct comparison of $[Ca^{2+}]_i$ in the two conditions was attempted in these studies.

Thus, differentiating the NPCs for three weeks (which is 35 days from the pluripotent stem cell stage), in medium containing 1.8 mM $Ca^{2+}{}_o$ resulted in an increase in spontaneous activity and a significant hyperpolarization. These effects were attenuated by co-incubation for the entire differentiation with inhibitors of $GABA_A$ receptors or blockers of L-type, N-type or R-type Ca^{2+} channels; consistent with the Ca^{2+} imaging data presented in Figure 1A, blockade of P/Q channels had no effect on the ability of raised $Ca^{2+}{}_o$ to augment spontaneous activity and to hyperpolarize the resting membrane potential (Figure 1C). Note that the potent Ca^{2+} channel opener, BayK 8644, was also able to enhance spontaneous activity and to hyperpolarize the neurons in standard $[Ca^{2+}]_o$ (Rushton *et al.*, 2013).

Chronic incubation of differentiating iPSCs with GABA evoked a significant increase in spontaneous activity and hyperpolarization at week 1. However, this augmentation in function was not further enhanced at week 2 (Figure 1D), suggesting that GABA might only be useful in promoting neuronal maturation early in the differentiation programme. The idea that GABA might provide the stimulus for $Ca^{2+}{}_o$ influx early in neuronal differentiation/maturation process, perhaps both *in vivo* and *in vitro*, is consistent with knowledge that ionotropic $GABA_A$ receptors are actually excitatory in prenatal

neurons. Thus, GABA binding elicits Cl^- exit and consequent depolarization (Ganguly *et al.*, 2001; Ben-Ari, 2002; Tozuka *et al.*, 2005). The postnatal transition from excitatory to inhibitory is a result of the developmentally modulated Cl^- equilibrium potential, which is itself controlled by the differential expression of Na^+:K^+:2Cl^--co-transporters (NKCC1) and K^+:Cl^--co-transporters (KCC2) (Ganguly *et al.*, 2001). Consequently, the $GABA_A$ phenotype (excitatory or inhibitory) of differentiating stem cells is a reliable functional readout for immature versus mature neurons. Human stem cell-derived neurons demonstrate robust $GABA_A$ currents and GABA-evoked $Ca^{2+}{}_o$ influx even at early stages of neuronal differentiation (Joannides *et al.*, 2007; Consortium., 2012) and GABA-dependent excitation has been shown to promote neuronal differentiation in several systems, including adult hippocampal stem/progenitors (Tozuka *et al.*, 2005) and neuroectodermal stem cells (Jelitai *et al.*, 2004). Therefore, it seemed likely that GABA might promote neuronal differentiation of iPSCs. Interestingly, although there is transition from excitatory to inhibitory GABA phenotype during early differentiation, ACM does not significantly alter the time-course of this event (Rushton *et al.*, 2013), suggesting that regulated $GABA_A$, NKCC1 and KCC2 expression do not contribute significantly to ACM-induced neuronal maturation *per se* (Rushton *et al.*, 2013). The GABA transition has also been well characterised during differentiation of human stem cells to cortical neurons (Livesey *et al.*, 2014); this study also showed time-dependent alteration in the expression of AMPA receptor composition. Whether this process is sensitive to $[Ca^{2+}]_o$ is not known (Livesey *et al.*, 2014).

Integrating GABA and raised $Ca^{2+}{}_o$ into a pro-synaptogenic differentiation protocol.

Having determined that maintaining $[Ca^{2+}]_o$ at 1.8 mM throughout the 3 week differentiation period and that GABA stimulation early in the process were both able to support enhanced neuronal function, as determined by observation of the frequency of spontaneous electrical activity and relative hyperpolarization, we began the rational design of a pair of media which would augment and/or accelerate functional maturation of iPSC-derived neurons. Medium 1 contained the high $[Ca^{2+}]_o$ together with GABA (used during the first week following plate-down of NPCs) whilst medium 2 contained just the high $[Ca^{2+}]_o$ (used from one week onwards). With the addition of brain-derived neurotrophic factor (BDNF) to both, these media were termed base media, to which various small molecules were added. For these particular particular experiments, pre-patterning to NPCs took 16 days, meaning the entire process, from pluripotent stem cell stage to mature neurons took 35-37 days

One of the major problems associated with neuronal differentiation from stem cells is the lack of synchronicity in the process. This means that neurons can be "born" from NPCs at almost any time

during the differentiation and this results in distinct lack of neuronal functional homogeneity. To address this issue, we employed inhibitors of both Notch signaling and the cell cycle checkpoint cyclin-dependent kinases, CDK4 and CDK6, in order to promote cell cycle exit (Telezhkin *et al.*, 2016). Blockade of Notch signaling with the γ-secretase inhibitor, DAPT, or CDK4/6 kinase activity with the selective inhibitor, PD0332991(Toogood *et al.*, 2005), significantly reduced the proportion of NPCs actively proliferating at 3 and 7 days post plate-down. In combination, these small molecules evoked synchronous, sustained cell cycle exit (Figure 2A, B) and enhanced neural differentiation, as shown by an increase in the proportion of cells expressing the neuronal marker, β-III-tubulin, and a marked reduction in the neural progenitor marker, nestin, at 7 days (Telezhkin *et al.*, 2016). These data suggested that these molecules should be added to the base media.

It is well known that $GABA_A$ receptor activation leads to Ca^{2+}-dependent CREB and ERK phosphorylation, which positively regulates neurogenic gene expression (Merz *et al.*, 2011; Wiegert & Bading, 2011). Furthermore, functional expression of neuronal voltage-gated Na^+ and K^+ channels during differentiation can also be affected by agents that elevate cAMP (Aglah *et al.*, 2008; Ravni *et al.*, 2008). Thus, medium 1, which already contained high $[Ca^{2+}]_o$ and GABA to drive the $GABA_A$-dependent increase in $[Ca^{2+}]_{i,}$ was supplemented with forskolin to raise intracellular $[cAMP]_i$, leading to CREB phosphorylation (Telezhkin *et al.*, 2016).

As GABA-dependent CREB phosphorylation has a transient developmental role in neurogenesis (Jagasia *et al.*, 2009), GABA and forskolin were removed from Medium 2, to encourage maturation during the later phase differentiation. Similarly, since the pro-neurogenic effects of Notch inhibition are only needed early in neurogenesis, and sustained γ-secretase inhibition can be deleterious (Shen, 2014), DAPT was also removed from medium 2.

A directed screen of growth factors and small molecules was then performed in order to identify compounds which enhance neuronal excitability. Little or no enhancement in excitability was observed with BDNF, FGF2, IGF1 or Activin A. However, the GSK3β antagonist, CHIR99021, did increase action potential firing, which prompted us to incorporate it into both media for further analysis. CHIR99021 has been previously demonstrated to promote post-mitotic nociceptor neural differentiation, although this was in combination with DAPT (Chambers *et al.*, 2012).

Functional consequences of neuronal differentiation in the enhanced media.

With the components of the two media complete, plated NPCs derived from several iPSC sources, derived using both integrating and non-integrating reprogramming vectors, were differentiated for one week in medium 1, and 2 more weeks in medium 2. Together, these two media are now termed SynaptoJuice (www.synaptojuice.co.uk). The potential benefits of using these media were determined by comparing their effects on electrical activity and neurotransmitter responses with those seen in base medium and standard differentiation medium (Telezhkin *et al.*, 2016). Differentiation in the enhanced media yielded the highest proportion of neurons demonstrating spontaneous electrical activity (Figure 3A, B), which was consistent with the data showing that such neurons displayed the most hyperpolarised membrane potentials and suggested that there was a more rapid transition from inactive to active state via evocation of relative hyperpolarization (Figure 3C). Such a notion is again entirely consistent with our preliminary observations that M-currents are developmentally regulated both *in vivo* and *in vitro* (Telezhkin *et al.*, 2014).

In terms of induced electrical activity, differentiation in enhanced media supported the highest proportion of neurons exhibiting action potential trains compared to those differentiated in base or standard media (Figure 4 and (Telezhkin *et al.*, 2016)); and the current injection *vs.* spike frequency plots showed that the enhanced media supported the greatest excitability (Telezhkin *et al.*, 2016). Differentiation in the enhanced media generated neurons with significantly shorter half-widths and larger overshoots, afterhyperpolarizations, spike amplitudes and depolarization rates (Telezhkin *et al.*, 2016). Surprisingly, neither voltage-activated Na^+ nor K^+ current densities were affected by the differentiation protocol. However, differentiation in the enhanced media generated neurons which displayed significantly altered Na^+ current activation/inactivation profiles; resulting in larger availability windows and a higher proportion of neurons with Vm values falling within those windows than did either base or standard media resulting in the probability of a neuron firing a spontaneous action potential being increased (Telezhkin *et al.*, 2016). These data suggested that the enhanced media improved functional maturation by facilitating neuronal hyperpolarization (to support higher spontaneous activity), and by increasing Na^+ current availability (to enhance regenerative action potential train activity).

To examine whether any set of media was able to support functional synaptogenesis, spontaneous miniature synaptic currents (minis), evoked post-synaptic currents, neurotransmitter-evoked changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and network activity were determined. Cells differentiated in standard medium for 3 weeks never exhibited miniature synaptic currents. Differentiation in base media only rarely supported the generation of GABAergic minis. In contrast, up to 75 % of neurons differentiated in the enhanced media exhibited large GABAergic and modest

glutamatergic minis. All cells differentiated in any of the media exhibited GABA-evoked currents, whereas there was a distinct enhancement in the proportion of cells exhibiting, and the magnitude of, NMDA-evoked currents in the enhanced media. These observations were supported by the demonstration of punctate, presumably synaptic, staining of NR1, NR2A and NR2B NMDA receptor subunits, co-registration of the pre- and post-synaptic markers, synaptophysin and PSD95 and the appearance of dendritic spines exclusively in the neurons differentiated in the enhanced media (Telezhkin *et al.*, 2016).

As the enhanced media appeared to improve the extent of functional neuronal maturation, weekly imaging of the neurotransmitter responses was performed in order to determine the time-course of this enhancement. From a very low level at the time of plating the NPCs, most of the cells from all three protocols responded to a depolarizing challenge at 1 week of differentiation and thereafter (Telezhkin *et al.*, 2016). However, from 2 weeks onwards, the magnitude of the responses was significantly lower for neurons differentiated using either standard or base media and this persisted until 3 weeks in standard medium (Telezhkin *et al.*, 2016). This was consistent with augmented expression of voltage-activated Ca^{2+} channels being supported by the enhanced media, and which has been previously documented to occur with ACM (Rushton *et al.*, 2013). The proportion of neurons responding, and the magnitude of the Ca^{2+} response, to glutamate/glycine increased during differentiation, but the enhanced media encouraged a more rapid augmentation which was delayed by a week in base medium and was never enhanced in standard differentiation medium (Figure 5A-C). Likewise, the proportion of cells responding, and the magnitude of the response, to GABA in low $[Cl^-]_o$ remained significantly smaller for standard and base media (Figure 5A-C). Furthermore, since the response to GABA in normal $[Cl^-]_o$ is potentially composed of both excitatory $GABA_A$ and facilitatory $GABA_B$ (Karls & Mynlieff, 2015) receptor responses, it seems reasonable to assume that our enhanced media, which largely abrogate this response to GABA in normal $[Cl^-]_o$ (Figure 5C), are promoting the transition from immature to mature $GABA_A$ phenotype and, possibly, causing a reduction in $GABA_B$ expression. As little is known about the ontogeny of $GABA_B$-dependent Ca^{2+} signaling in developing neurons, but it is known that $GABA_A$ responses transition from excitatory to inhibitory, loss of $GABA_B$ might also be an indication of maturity in pluripotent stem cell-derived neurons. This, and the precise timing of the functional switch in $GABA_A$ phenotype, which are promoted by the enhanced differentiation media are currently under investigation.

Finally, evidence that the enhanced media supported the formation of excitatory synaptic networks whose activity was being tonically dampened by inhibitory GABAergic tone was provided by experiments using 24-well microelectrode array (MEA) plates which showed that large and maintained increases in activity could be evoked by the addition of gabazine, a $GABA_A$ receptor

inhibitor (Figure 5D). Furthermore, glutamate was able to stimulate activity whilst tetrodotoxin completed abolished activity in all wells (Telezhkin *et al.*, 2016).

Comparison with alternative differentiation media and protocols

Thus, our recent published studies support the notion that forced cell cycle exit is able to synchronize neuronal differentiation. That simple manoeuvre, coupled to simulating fetal conditions by increasing $[Ca^{2+}]_o$ throughout whilst transiently generating a GABA excitatory drive and tonically applying known pro-synaptogenic small molecules (CHIR 99021 and forskolin), results in rapid functional maturation of neurons with highly homogeneous response profiles. Although there has been sustained activity for over a decade aimed at producing neurons from a variety of stem cell sources, it is only recently that there has been brisk and highly competitive progress towards generating demonstrably functional neurons exhibiting characteristically appropriate synaptic properties *in vitro*. There have been several other carefully performed recent studies which have demonstrated maturation by a variety of functional endpoints (Song *et al.*, 2013; Livesey *et al.*, 2014; Telias *et al.*, 2014; Bardy *et al.*, 2015). The study by Song and colleagues analysed the electrophysiological time-course of pluripotent stem cell-derived neuronal differentiation (Song *et al.*, 2013). Although their data are in broad agreement with ours, the homogeneity of the neurons with respect to repetitive action potential train generation are more similar at 3 weeks to those generated by our standard differentiation medium. Even after a further week, the Song protocol still only supported repetitive firing in under 50 % of the neurons, likely due to the lack of synchronization of the differentiation programme that our enhanced media afford early in neurogenesis. Furthermore, their induced action potentials have lower spike amplitudes, slow depolarization rates and long half-widths; again more reminiscent of those we see in standard differentiation medium. The Livesey protocol (Livesey *et al.*, 2014) used several important readouts of neuronal maturation, including $GABA_A$ phenotype (Ganguly *et al.*, 2001). They observed $GABA_A$ response transition from excitatory to inhibitory during the 7 week differentiation, which was due to progressive reduction in chloride equilibrium potential (E_{Cl}) as the expression of NKCC1 and KCC2 were reduced and enhanced, respectively (Rivera *et al.*, 1999; Yamada *et al.*, 2004); no data were available between 1 and 5 weeks. With our enhanced media, inhibition of GABAergic signaling with gabazine resulted in a dramatic

increase in neuronal network activity which suggests that even by 3 weeks, synaptic GABA was inhibitory across the network. In the Telias protocol (Telias *et al.*, 2014) neurons differentiated from human ESCs demonstrated significant maturation properties by 3 weeks, including a high proportion of neurons with train activity. However, input resistance was high and individual action potential amplitudes were very low compared to neurons differentiated in our enhanced media. Their use of DAPT was encouraging and might have been expected partially to synchronise the neuronal maturation. However, there were no data presented regarding potential synchronization and it might be more prudent to use both PD0332991 with DAPT, as we have now shown (Telezhkin *et al.*, 2016). Finally, and of particular note, is the exceptionally elegant study of Bardy and colleagues who, using an evidence-based approach, systematically manipulated the composition of their medium in order to optimise neuronal functional readouts (Bardy *et al.*, 2015). This medium supported high levels of synaptic function in both primary neurons and in differentiated stem cells. Now termed BrainPhys (Stem Cell Technologies), this medium contains, in contrast to SynaptoJuice, adult $[Ca^{2+}]_o$. Compared to our enhanced media, activity appears to take longer to develop, but because the authors have shown that it supports neuronal activity for many weeks longer than SynaptoJuice has been assayed so far, it could well be a perfect candidate for the "third" medium for use after our enhanced media, in order to support long-term aging of neurons; something that will be important for successful modelling of late-onset neurodegenerative diseases.

Figure Legends

Figure 1. *Potential mechanisms of ACM-evoked enhancement of neuronal function.*

A. Fura-2 derived relative intracellular Ca^{2+} concentration measurements for iPSC-derived NPCs after 1 week of differentiation in either standard differentiation medium (SDM, upper trace) or (ACM, lower trace). Ca^{2+} influx was evoked by depolarization using 50 mM KCl in the absence and presence of 10 μM nifedipine (L-type Ca^{2+} channel blockade), 100 nM conotoxin (N-type Ca^{2+} channel blockade), 100 nM agatoxin (P/Q Ca^{2+} channel blockade) or 100 nM SNX482 (R-type Ca^{2+}channel inhibition).

B. Bar graphs comparing the proportion of neurons firing spontaneous action potential (sAPs, upper bars) and mean resting membrane potential (V_m, lower bars) following 3 weeks of differentiation in

ACM alone, or ACM with 10μM bicuculline, 2 μM nifedipine, 100 nM conotoxin or 100 nM SNX482.

C. Comparison of the proportion of neurons firing sAPs (upper bars) and mean Vm (lower bars) following 3 weeks of differentiation in 1.2 or 1.8 mM $[Ca^{2+}]_o$ and 1.8 $[Ca^{2+}]_o$ in the presence of 10μM bicuculline, 2 μM nifedipine, 100 nM conotoxin or 100 nM agatoxin.

D. Comparison of the proportion of cells firing sAPs (upper bars) and mean Vm (lower bars) following 1 and 2 weeks of differentiation in SDM with and without 300μM GABA. Different from control, ***p < 0.001. Adapted from (Rushton *et al.*, 2013).

Figure 2. *Synchronous cell cycle exit and differentiation are by promoted γ-secretase and cyclin dependent kinase 4/6 inhibition.*

Exemplar fluorescence micrographs (upper) and means cell counts (lower) of MKI67 immunoreactivity (green) and cell nuclear staining with DAPI (blue) of neural progenitor cells (NPCs) 3 (A) and 7 days (B) post-plating in base medium with no additions (Control), or base medium supplemented with 2 μM PD0332991 or 10 μM DAPT, or both.. Different from control, ***p < 0.001, **p < 0.01, *p < 0.05. Scale bars = 100 μm. Adapted from (Telezhkin *et al.*, 2016)

Figure 3. Enhanced differentiation media (*SynaptoJuice)* augment *the generation of neurons which exhibit spontaneous action potentials*.

A. Typical current-clamp recordings (I = 0 pA) illustrating the three separate types of activity which were observed spontaneously in the neurons: spontaneous action potentials (*sAP-full*, upper); incomplete initiation of spontaneous action potentials (*sAP-incomplete initiation*, middle), and; no spontaneous action potentials (sAP-*None*, lower). Note that each sAP type is also the modal activity for neurons differentiated in SynaptoJuice, base medium and standard differentiation medium, respectively.

B. Proportions of neurons which demonstrated each of the individual sAP characteristics when NPCs were differentiated into neurons for 3 weeks in the enhanced media (*SynpatoJuice*), base medium and standard differentiation medium (i.*e.* 35 days from pluripotent stem cells).

C. Mean resting membrane potentials (Vm) of neurons differentiated for 3 weeks in SynpatoJuice, base medium and standard differentiation medium. ***p < 0.001, ****p < 0.0001. Time bar applies to all traces. Adapted from (Telezhkin *et al.*, 2016).

Figure 4. Enhanced differentiation media (*SynaptoJuice) augment the generation of neurons exhibiting induced action potential train.*

A. Exemplar current-clamp recordings (I = 0 pA) illustrating the five separate types of induced activity which were observed in the neurons: Train of induced action potentials (*iAP-Train*); Single action potential with incomplete initiation of action potential trains (*iAP-Incomplete initiation*); Single action potential (*iAP-Single*); Incomplete initiation of single action potential (*iPS-Incomplete single*), and; no induced action potential (*iAP-None, lower*). Time bar applies to all traces. Adapted from (Telezhkin *et al.*, 2016).

B. Proportions of neurons which demonstrated each of the individual iAP characteristics when NPCs were differentiated into neurons for 3 weeks in the enhanced media (*SynpatoJuice*), base medium and standard differentiation medium (*i.e. for* 35 days from pluripotent stem cells).

Figure 5. *Enhanced differentiation media (SynaptoJuice) improve time-dependent maturation of post-synaptic neurotransmitter responses and supports regulatable network activity.*

A. Proportion of neurons displaying Ca^{2+} influx in response to 60 mM KCl (KCl), 300 μM glutamate/30 μM glycine (Glu/Gly) and 300 μM GABA in 7.5 mM chloride (Low Cl^-/GABA) following differentiation for 3 weeks of NPCs (*i.e.* 35 days from pluripotent stem cells) from NPCs in SynpatoJuice, base medium, and standard differentiation medium.

B. Mean magnitude of Ca^{2+} influx, expressed as change in fluorescence from baseline (Δ Fluorescence) of the same neurons used for the analysis shown in panel A. ***p < 0.001, **p < 0.01, *p < 0.05.

C. Exemplar fura-2-dervied intracellular Ca^{2+} recordings from individual neurons of the cohort shown in panels A and B. Note the response to 300 μM GABA alone (GABA) was large in neurons in standard differentiation medium, small in base medium and often not detectable in enhanced differentiation media.

D. Exemplar recording of spontaneous network spike rate before (*left*) and following (*right*) the addition of 5 μM gabazine to one of 24 wells in a multi-well microelectrode array. Note the split time axis for addition of drug. Single unit activity is shown as an insert. Neurons were differentiated from NPCs for 3 weeks in enhanced differentiation medium (i.e. 35 days from pluripotent stem cells. Adapted from (Telezhkin *et al.*, 2015).

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Figure 1

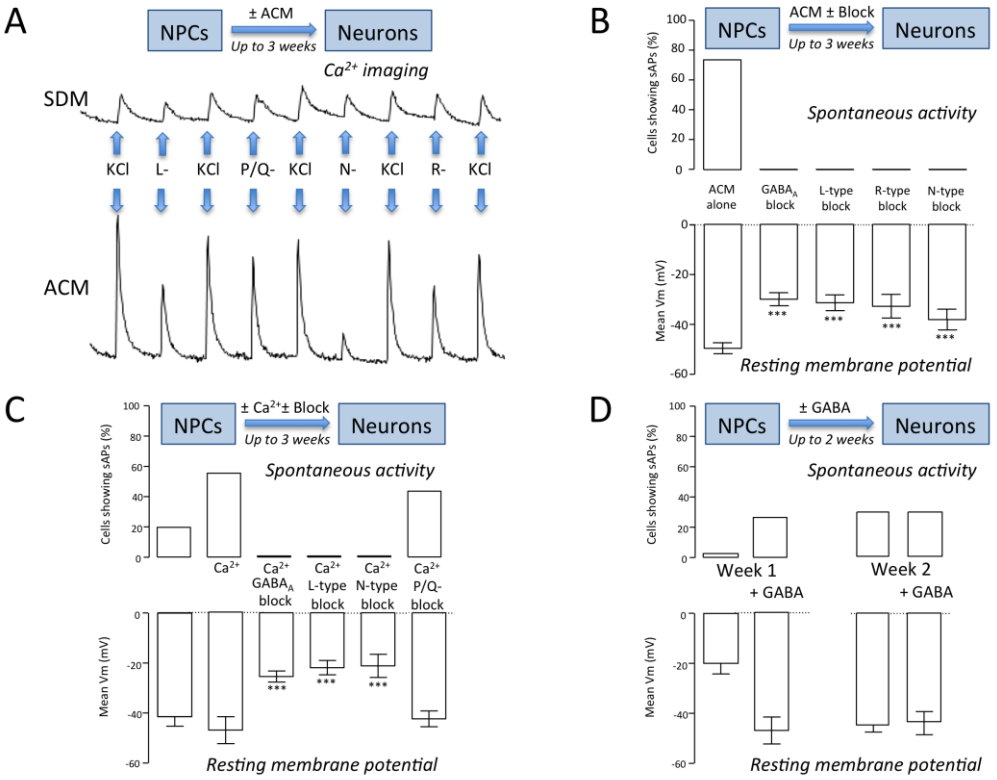
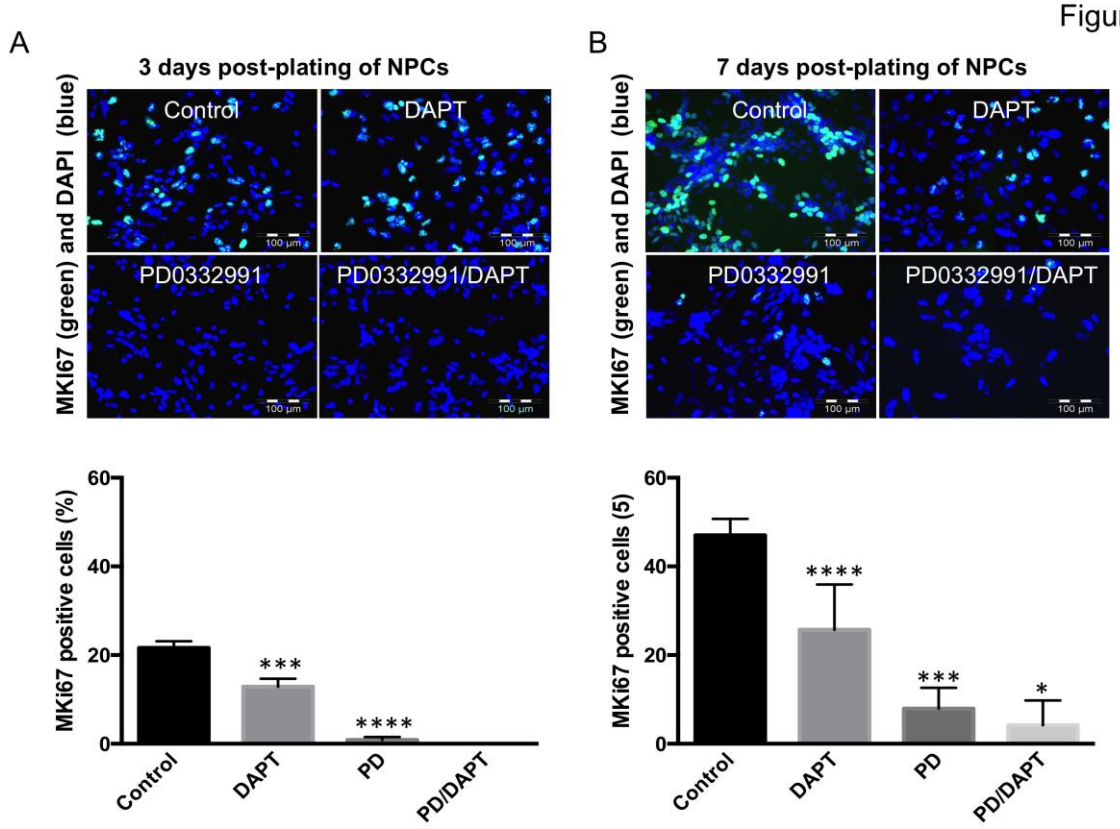


Figure 2



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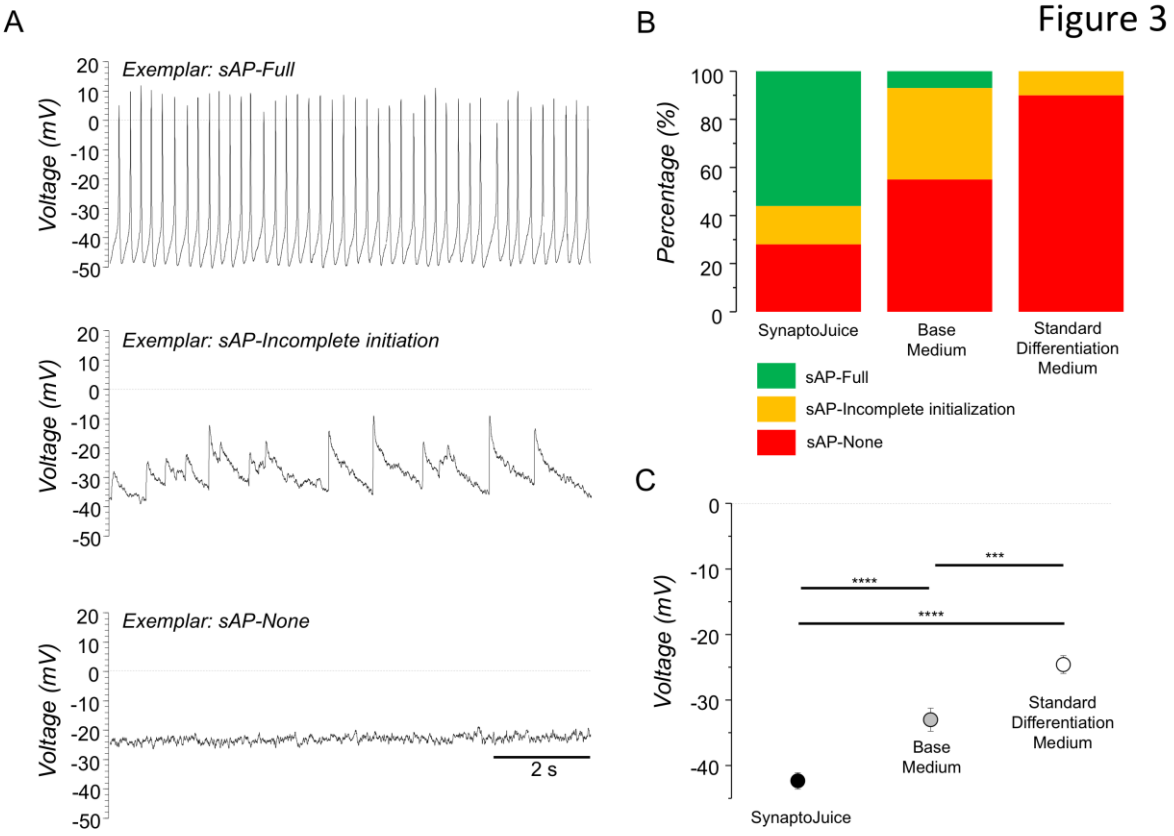

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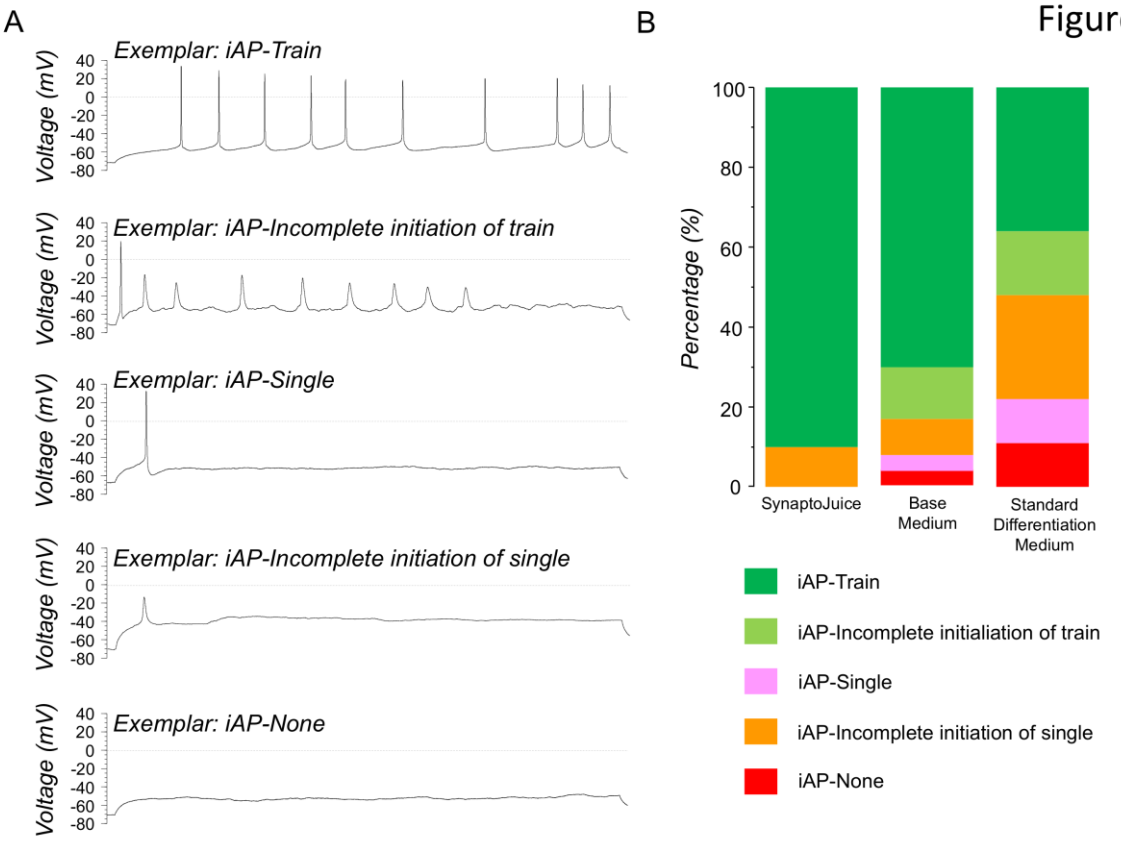

Figure 5